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Cellular Differentiation and Nitrogenase Activity in the Cyanobacterium *Anabaena*

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Abstract. Nitrogenase activity at periods of differentiation of heterocysts and akinetes was assayed by the acetylene reduction technique. There was no nitrogenase activity in ammonium-grown, non-heterocystous *Anabaena* sp.; the activity appeared only after a lag-phase of about 17–21 h after the ammonium-grown culture had been transferred to medium free of combined nitrogen. This activity started appearing as the proheterocysts were developing to mature heterocysts. Maximum nitrogenase activity was attained with exponential phase of culture and mature heterocysts. This activity gradually decreased with the differentiation of akinetes. Only insignificant nitrogenase activity was observed in old cultures in which most cells had matured into akinetes.

The blue-green algae (cyanobacteria) are procaryotic, mostly photoautotrophic organisms capable of fixing dinitrogen. These organisms provide good examples of elaborate morphological and physiological differentiation of one cell type to another. They are the organisms chosen for the study of cellular differentiation in a simple situation, mainly because of their geometrical simplicity and versatile growth habit.

Of the many problems presented by this group, that offered by cellular differentiation and dinitrogen fixation has attracted considerable attention. The majority of these algae contain three types of cell *i.e.*, vegetative cell, heterocyst and akinete (spore). Heterocysts and akinetes are the structures with specific structural and biochemical properties suggestive of their role as organs of nitrogen fixation and perennation respectively (WOLK and WOJCIUCH 1971, YAMAMOTO 1975, THOMAS *et al.* 1977). Heterocysts, formed at specific loci in the filament, act as main sites of nitrogenase activity (FAY *et al.* 1968, THOMAS and DAVID 1972, FLEMING and HASELKORN 1973). Akinetes are commonly formed in some heterocystous algae at specific positions in the filaments in relation to the heterocysts. These are thought to be perennating structures because of their increased resistance toward unfavourable environmental conditions (YAMAMOTO 1975). In this communication, we describe the differentiation of these cells and nitrogenase activity at different stages of their development.

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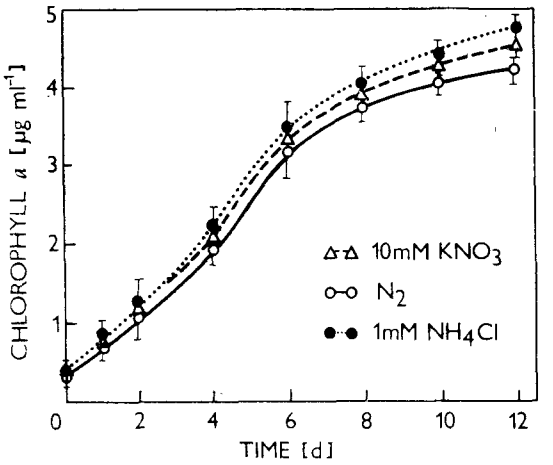

Fig. 1. Growth pattern of *Anabaena* in basal and combined nitrogen supplemented medium.

MATERIAL AND METHODS

Organism and Cultural Conditions

Anabaena sp., a filamentous nitrogen-fixing alga forming heterocysts and akinetes was used for these studies. It was isolated in a clonal axenic culture from paddy fields of Banaras Hindu University in modified Hughes' medium (ALLEN and STANIER 1968), free of combined nitrogen and supplemented with 1% Difco Bacto agar (m/v); citrate and phosphate were added to the medium after autoclaving separately to avoid precipitation. Cultures were illuminated with illuminance of about 1.5 klx for 14h/10h light/dark cycles at 27 ± 2 °C. Liquid medium was used for growth, nitrogenase assay and differentiation studies. Inoculum was prepared from exponentially growing vegetative cells (non-heterocystous) of the alga in 1 mM NH_4Cl by repeated centrifugation and washing with sterilized double distilled water. A homogeneous suspension was made by glass beads and equal inocula were added to each tube or plate for different experiments. Growth was measured by extracting methanol-soluble pigments. Heterocyst and akinete frequency was calculated as the number of heterocysts per hundred vegetative cells and akinete-heterocyst connections per hundred heterocysts respectively. TTC reaction was tested during different stages of the life cycle of the alga using 0.1% solution of 2,3,5-triphenyl tetrazolium chloride.

Nitrogenase Assay

Nitrogenase activity was measured by the acetylene reduction technique (STEWART *et al.* 1967). Assay was performed in calibrated triplicate serum bottles of about 7.6 cm^3 capacity. The acetylene concentration was kept at 10% and 2 ml cell suspension was routinely injected in each bottle. Reactions were run for 30 min at 27 ± 2 °C and 3.0 ± 0.2 klx. During incubation, the serum bottles were shaken continuously on a shaker. Reaction was terminated by injecting 0.8 ml of 15% trichloroacetic acid. Ethylene produced in the vessel was analysed in a CIC gas chromatograph (Baroda) using Porapak-R column. Standard was prepared using pure ethylene. Chlorophyll was measured according to TALLING and DRIVER (1963). Typical nitrogenase activity in an exponentially growing culture lies in the range of 10.6—17.2 nmol $C_2H_4.h^{-1}$ $bottle^{-1}$.

TABLE 1

Nitrogenase activity (acetylene reduction) at different stages of heterocyst development in *Anabaena* sp.

Time after transfer to basal medium [h]	Type of cells	Acetylene reduction [nmol C_2H_4 . h^{-1}(μg chl.)$^{-1}$]
0	Vegetative cells (VC)	—
12	VC, rarely proheterocyst, (PH)	—
20	VC, PH, rarely heterocyst (H)	0.02 ± 0.01
28	VC, H	0.13 ± 0.02
36	VC, H	0.26 ± 0.07
48	VC, H	1.15 ± 0.21
72	VC, H	1.09 ± 0.24

RESULTS

Fig. 1 shows the growth response of the alga in basal medium (N2 medium) and with addition of inorganic combined nitrogen sources. Ammonium chloride inhibited both heterocyst formation and akinete differentiation but enhanced algal growth. Nearly similar response was shown by potassium nitrate. The heterocyst frequency varies around 5.0 per cent. Akinetes were formed after 6—8 d and sometimes attained a frequency of 95—100% after 10—12 d. The mean generation time of the alga in basal medium is 52 ± 2 h.

Heterocysts appeared at specific loci at regular intervals in the filaments, when ammonium-grown non-heterocystous filaments were transferred to basal medium. There was no nitrogenase activity in the ammonium-grown non-heterocystous culture. After 17—21 h of transfer, some cells (called proheterocysts) at regular intervals in the filament became enlarged and lost some pigments. Nitrogenase activity started after this stage (Table 1). These enlarged cells (proheterocysts) become hyaline after six or more hours and two polar nodules appeared in each cell (Table 2). The thick wall was clearly observable under a light microscope. Maximum nitrogenase activity was found at this stage along with higher frequency of heterocysts (Fig. 2A).

Akinetes are generally formed adjacent to heterocysts after the exponential phase of growth (Table 3). This position of akinete is generally quite specific though exceptions do occur in some cases. Akinetes are large, thick-walled granulated cells. Shortly before the commencement of their development, there is an appreciable rise in the nitrogenase activity level. But this production declines as the differentiation of akinete progresses. In later phases of development, there is an insignificant amount of acetylene reduction and the culture contains about 95—98 per cent of akinete-heterocyst connections

TABLE 2

Timing of developmental processes and nitrogenase in *Anabaena* sp. after deprivation of ammonia

	Time of appearance [h]
Proheterocysts	12 ± 2.0
Heterocysts	20 ± 3.0
Akinetes	140 ±12.0
Nitrogenase	18 ± 3.0

(Table 4, Fig. 2B). However, the nitrogenase activity level increases again with the germination of akinetes in the fresh basal medium. We also tried to test the reducing ability of the differentiated cells at different stages with triphenyl tetrazolium chloride (TTC) (Table 5). Heterocysts of three day old filaments showed a dense pink formazan formation while the vegetative cells showed very poor reaction and then lysed. In filaments with young akinetes, only the heterocysts showed the reaction. The formazan formation

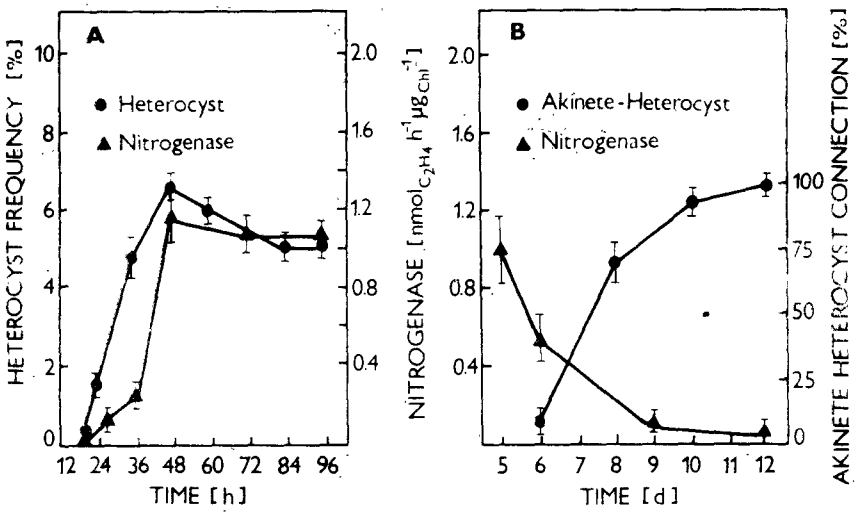

Fig. 2. Cellular differentiation and nitrogenase activity in *Anabaena*. — (A) Heterocyst formation; (B) Akinete formation.

was very poor in heterocysts of sporulated filaments. Akinetes did not form any formazan crystals. These cells can perennate for months together in the dark and when transferred to growth-conducive conditions, give rise to filaments.

DISCUSSION

When the *Anabaena* sp. culture is transferred into a medium free of combined nitrogen, proheterocysts and then heterocysts are formed in an ordered sequence. Simultaneously with the appearance of heterocysts in the culture, the activity of nitrogenase starts. This supports the findings of

TABLE 3
Dimensions of vegetative cells, heterocysts and akinetes in *Anabaena* sp.

Type of cell	Dimensions [µm]	
	Length	Breadth
Vegetative cell	3.2 . . . 4.8	3.0 . . . 3.6
Heterocyst	5.5 . . . 6.8	5.0 . . . 5.9
Akinete: Phosphate deficient medium	12.0 . . . 16.0	5.2 . . . 7.8
Phosphate sufficient medium	15.5 . . . 19.8	5.2 . . . 7.8

TABLE 4
Nitrogenase activity in cultures of *Anabaena* in relation to time

Age of culture [d]	Type of cells	Acetylene reduction [nmol C_2H_4 . h^{-1}(μg chl)$^{-1}$]
3	Vegetative cells (VC) Heterocysts (H)	1.05 ± 0.19
6	VC, H, Akinetes (A)	0.53 ± 0.15
9	VC, H, A	0.10 ± 0.06
18	VC, H, A	0.02 ± 0.01
50	VC, H, A	—

BRADLEY and CARR (1976) in *Anabaena cylindrica*. The rate of acetylene reduction increases with maturity of heterocysts and maximum activity is observed when the frequency of heterocysts is highest. Heterocysts lack photosystem-II activity and have an active nitrogenase enzyme system. SUTHERLAND *et al.* (1979) found that the slowly declining rate of acetylene reduction during akinete differentiation became negligible as the akinetes matured. In *Anabaena*, we have also observed higher rates of acetylene reduction before the beginning of akinete formation, but as the akinete formation progressed, this activity fell (Table 4).

The faster reduction of acetylene before akinete formation may be due to transportation of more nitrogenous compounds from heterocysts to adjoining vegetative cells, thus causing some nitrogen deficiency within the heterocysts. These adjoining cells first transform into akinetes with a distinct store of cyanophycin granules. This rate of acetylene reduction decreases at the stage of akinete maturation possibly due to lack of energy sources since there are no vegetative cells in direct connection with the heterocyst for the provision of photosynthates (WOLK 1968). Perhaps because of this reason, photosystem-II pigments may again appear in old heterocysts, as suggested by THOMAS (1972).

Till now acetylene reduction rate has been thought to be driven by PS I reaction only, consistent with almost total absence of phycobiliproteins in the heterocyst. These cells lack oxygen evolving PS II and are thus suitable sites for harbouring the nitrogenase. FAY (1970) found the best photo-

TABLE 5
Formazan crystal formation by TTC reaction at various stages of life cycle of *Anabaena* sp.

Cell age [d]	Cell type	Observation after 6 min	Observation after 20 min
3	Heterocyst	pink crystals	bigger crystals
	Vegetative cell	absent	small crystals and lysis
6	Heterocyst	small crystals	small crystals
	Vegetative cell	absent	small crystals and lysis
	Akinete	absent	absent
15	Heterocyst	smaller crystals	less crystals
	Vegetative cell	absent	less crystals and lysis
	Akinete	absent	absent

stimulation of acetylene reduction rate at 675 nm, the wavelength most absorbed in pigment system I and little effective in oxygen evolution. This suggested that PS I is involved actively in nitrogen fixation probably to supply ATP through cyclic photophosphorylation. But TYAGI (personal communication) has shown the involvement of the usual PS II pigments (phycobiliproteins) in acetylene reduction as well as their presence in heterocysts. Oxygen evolved in the presence of PS II pigments may be taken up by the specialised lipids of the heterocyst envelope. We have also got enzyme activity for acetylene reduction in the sporulated cultures but it is very low.

As the akinetes generally form next to heterocysts, the heterocyst may be regulating the pattern of akinete formation (WOLK 1966). However, we sometimes observed one or two akinetes being formed away from heterocysts or being formed in filaments devoid of heterocysts. Thus the role of the heterocyst in inducing akinete formation in *Anabaena*, as also in others (AHLUWALIA and KUMAR, communicated), still seems enigmatic.

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